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Effectiveness of Azorizobial strains isolated from *Sesbania rostrata*

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ABSTRACT

Isolation and characterization of bacteria were done from the root nodules of African dhaincha (*Sesbania rostrata*) to study their effects on growth parameters, nodulation and N uptake. Five isolates were obtained from African dhaincha and were identified them as *Azorhizobium* on the basis of their colony, morphological and biochemical characteristics. A pot experiments were conducted by using 6 treatments, comprising 5 isolates of *Azorhizobium* and an uninoculated control to evaluate the performance of the isolates. The experiment was laid out in a Completely Randomized Design (CRD) with 3 replications. *Azorhizobium* inoculation gave statistically higher values for all the parameters *viz.* plant height, leaf number plant^{-1}, dry weight of nodule, dry matter yield, N content of shoot (%), total N in shoot over uninoculated control (T0). The highest values of all the parameters except stem nodule number plant^{-1}, root nodule weight and N content of shoot were obtained with the treatment T4 (SR-R-4). The total dry matter yield of African dhaincha ranged from 1217 mg plant^{-1} noted in the uninoculated control (T0) to 5483 mg plant^{-1} recorded for the treatment T4 (SR-R-4). The total dry matter yields were increased by 138, 219, 273, 351 and 290% over control (T0) due to T1 (SR-R-1), T2 (SR-R-2), T3 (SR-R-3), T4 (SR-R-4) and T5 (SR-R-5), respectively. Total N uptake by shoot of African dhaincha had been influenced significantly due to different treatments and the corresponding percent increases in total N uptake by shoot were 359, 496, 429, 629, and 490, respectively, due to T1 (SR-R-1), T2 (SR-R-2), T3 (SR-R-3), T4 (SR-R-4) and T5 (SR-R-5). Considering all the growth parameters, nodulation, dry matter yield, N content of shoot and total N uptake by shoot, it may be inferred that the isolate SR-R-4 showed the best performance. The isolates SR-R-5 and SR-R-3 were also found promising.

Keywords: *Azorhizobium*, *Sesbania rostrata*, nodulation, dry matter yield

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1 Introduction

Sustained agricultural production has gradually become the cornerstone of development policies in tropical, sub-tropical and Mediterranean countries. From the mid-sixties onward, the increase in agricultural production was based on the introduction of new high yielding varieties of crops, which required the application of large amounts of chemical fertilizers and pesticides. This policy, which underlay the green revolution, was successful in a number of Asian and South American countries. But, it would not bring desirable benefit to many of the less-favoured areas where adequate fertilizers are not available and soils are very deficient in nutrients, especially nitrogen (N) and phosphorus (P) (Basu and Kabi, 1987).

Bangladesh soils are typically very low in nitrogen. Nitrogen deficiency arises mainly due to rapid decomposition of organic matter content induced by warm climate, losses of N due to denitrification, leaching and high removal by intensive cropping with modern varieties. But the continuous application of chemical fertilizers has become detrimental to soil fertility. Consequently, the use of biofertilizers has increasingly been gaining global attention as one of the practices to restore and maintain soil fertility (Bhuiya et al., 1995).

As a legume crop, African dhaincha (*Sesbania rostrata*) has the unique ability to fix and utilize atmospheric nitrogen (N_2) for its own growth and at the same time, enrich soil fertility by increasing soil organic matter status. Such fixation of N_2 takes place through a biological process *i.e.* symbiotic association between *Sesbania* root and *Rhizobium* bacteria. The N fixation potential of *S. rostrata* has already been established and estimated as 176 kg N ha^{-1} in a period of 56 d (Furoc et al., 1985). In favourable environment, it can fix 70–458 kg N ha^{-1} from the atmosphere within 45–65 d after sowing (Ladha et al., 1989). Its use increases soil's capacity to absorb nutrients and improve soil structure and microbial activities (Zaman et al., 1994; BRRI, 1996). In addition, it can extract nutrients from deep soil layers, use insoluble or fixed forms of phosphorus and make them available to the succeeding crops due to its extensive and deep root system. Bhuiyan and Zaman (1996) reported that one ton dry weight of *S. aculeata* can supply 33.2, 13.6, 14.0 and 16.2 kg N, K, Ca and Mg, respectively. Increased nodulation and high dry matter accumulation of *Sesbania* due to *Rhizobium* inoculation have been documented by several workers (Tiwari et al., 1980; Furoc et al., 1985; Meelu et al., 1985). However, research work on the seed inoculation of *Sesbania* for effective nodulation, growth and biomass production and N_2 fixation received less attention in our country. Nevertheless, it is necessary to identify the effective strains in terms of nodulation and N-fixation ability of the host crop.

Keeping the above points in view, the present study was undertaken to isolate *Azorhizobium* spp. from African dhaincha (*S. rostrata*) root nodules and to study their effects on growth parameters, nodulation, dry matter production of and total N uptake by African dhaincha.

2 Materials and Methods

2.1 Isolation and characterization of *Azorhizobium*

Mature and large sized nodules were collected from African Dhaincha plants (*S. rostrata*) grown in Agronomy Field Laboratory of Agronomy Department, Bangladesh Agricultural University, Mymensingh. Nodules were washed in tap water to remove gross soil contamination. The nodules were immersed briefly (5 sec) into 95% ethanol. After that, nodules were put into petri dishes containing 0.1%-acidified mercuric chloride and left for 4 min. Then, nodules were rinsed in five changes of sterile water. Nodules were then transferred to a sterile petri plate and crushed individually with fine pointed sterile forceps to produce turbid suspension. One loopful of the suspension was streaked out on yeast-mannitol agar (YMA) plates (K_2HPO_4 0.5 g, $MgSO_4$ 0.2 g, NaCl 0.1 g, mannitol 10 g, yeast water 100 mL, distilled water 900 mL). The plates were then incubated at 28 °C and checked for typical colonies of *Azorhizobium* along the streak lines.

Well-isolated single colonies were picked off and restreaked on clean plates to obtain pure cultures. The form, margin, surface, diameter, optical characteristics, consistency and pigmentation of the colonies were studied on YMA media according to Harry and Paul (1972). Shape of the bacteria was examined by direct staining with crystal violet solution. Gram reaction was examined by modified Hucker and Conn (1923). Motility was examined by hanging drop method. Congo red dye absorption, starch utilization and pH were also performed to detect *Azorhizobium* (Dreyfus et al., 1985).

2.2 Pot study

The pot study was carried out in the Net house of the Department of Soil Science, Bangladesh Agricultural University, Mymensingh to study the effects of *Azorhizobium* inoculation on *S. rostrata*. The experiment was laid out in a Complete Randomized Design (CRD) with 6 treatments and 3 replications. The treatments were: T0 (Control), T1 (SR-R-1), T2 (SR-R-2), T3 (SR-R-3), T4 (SR-R-4) and T5 (SR-R-5). Measured quantity of 0.5 kg soil and sands mixture was used in each pot. The total number of soil and sands mixture filled pots was 18. The experimental soil was collected from Horticulture Farm of BAU which belongs to Sonatola series of Old Brahmaputra Floodplain (AEZ-9) and sands were collected from the adjacent riverside of the river Old Brahmaputra. Air-dried soil was ground to pass through a 2 mm sieve and the sands were also passed through the 2 mm sieve. Soil and sands were mixed at 1:1 ratio. Soil and sands mixture was sterilized in an autoclave at 121 °C and

15 PSI for 20 min. The pots were also sterilized by wiping with 40% alcohol (formalin solution).

2.3 Plant analysis

The shoot and root samples were ground by a grinding mill to pass through a 20-mesh sieve and stored in paper bags in desiccators. Total N content in plant samples were determined by Kjeldahl method. Catalyst mixture (K_2SO_4:$CuSO_4$.$5H_2O$:Se = 10:1:0.1) and 30% H_2O_2 and conc. H_2SO_4 were used for digesting plant samples. Estimation of N in the digest was done by distillation with 40% NaOH followed by titration of the distillate trapped in H_3BO_3 with 0.01 N H_2SO_4 (Page et al., 1989).

2.4 Statistical analysis

The collected data were analyzed statistically to test the significance and *F*-test was done to compare the treatment effects. The mean comparisons of the treatments were evaluated by Duncun's Multiple Range Test (DMRT) (Gomez and Gomez, 1984).

3 Results

3.1 Isolation and characterization of *Azorhizobium* isolates

The colonies of *Azorhizobium* isolates from stem nodules of *S. rostrata* appeared on Yeast Mannitol Agar (YMA) media within 6-7 d of inoculation. Five Azorhizobial isolates were obtained on YMA plates and they were designated as SR-R-1, SR-R-2, SR-R-3, SR-R-4 and SR-R-5. The colony characteristics of rhizobial isolates of African dhaincha did not vary widely. The isolates produced dull-white colonies on YMA media with entire margin and their forms were circular. Diameter of the colonies of African dhaincha isolates varied from 0.8 mm to 1.2 mm. Their surface was smooth and the optical characteristics of the colony were found translucent with viscous consistency (Table 1). All the African dhaincha isolates were also rod shaped, Gram negative and poorly motile (Table 1). The rhizobial cells appeared as transparent bodies in a grey background studied with negrosine. Similar results were also found by Vincent et al. (1980).

Three biochemical tests e.g. congo red dye absorption, starch utilization and pH test were done in the laboratory to study the biochemical characteristics of different African dhaincha isolates. Results recorded in Table 1 indicate that the isolates SR-R-3 and SR-R-4 absorbed congo red dye moderately but the isolates SR-R-1, SR-R-2 and SR-R-5 absorbed the dye slightly. In general, the rhizobia absorb the dye weakly whereas many other bacteria take up the dye strongly (Vincent, 1970). Results in Table 1 also indicate that all the isolates of *S. rostrata* failed to utilize the starch and produced alkali. This result has been supported by Odee et al. (2002). Characteristics of the isolates clearly indicated that they were *Rhizobium*. As they produced nodules on the stem, so they were *Azorhizobium*.

3.2 Effect of isolates on *S. rostrata*

3.2.1 Plant height

The application of *Azorhizobium* isolates significantly increased plant height (Fig. 1). After 30 d of sowing (DAS), in *S. rostrata*, plant height ranged from 34.1 cm to 63.3 cm plant^{-1}. The highest plant height of 63.3 cm plant^{-1} was obtained with the treatment T5 followed by the treatment T4, T2 and they were statistically identical but significantly different from the rest of the treatments. Treatments T1 and T3 were statistically similar but they both were significantly taller than T0 (control). At 60 DAS, the isolate SR-R-4 produced the tallest plant (113.5 cm) which was statistically different from the isolates SR-R-2 and SR-R-5. After 60 d of sowing, plant height ranged from 68.7 cm to 113.5 cm plant^{-1}. The better growth in treatment T4 could be due to effective N_2-fixation by the plants that produces from the microbial relationship between plants and microbes (Dahmardeh, 2013; Ella, 2006).

3.2.2 Leaf production

Data on leaf number plant^{-1} as affected by various treatments are presented in Fig. 1. The treatments had significant influence in producing leaves. Leaf number increased with the days after sowing. The effect of different types of *Azorhizobium* inoculation on leaf number in *S. rostrata* was significantly superior to no inoculation *i.e.* control. Here, leaf number ranged from 4.0 to 9.3 plant^{-1} at 30 DAS (Fig. 1). The highest number of leaf was obtained with the treatment T6 which was followed by T4 and then T2 and T3 all of which were statistically identical. Again, T5, T4, T2 and T3 differed significantly from T1 and T0 (control). The lowest number of leaf plant^{-1} was obtained with the treatment T0 (control) which was statistically inferior to all of the treatments. At 60 DAS, leaf number ranged from 15.0 to 24.7 plant^{-1} (Fig. 1).

3.2.3 Nodule formation

There was significant effect of inoculation with different Azorhizobium isolates in producing the stem nodulation of *S. rostrata* (Fig. 2). The results show that the isolates SR-R-1, SR-R-2, SR-R-3, SR-R-4 and SR-R-5 gave significantly higher number of nodules on stem over control. The highest number of stem nodules was 41.0 plant^{-1} recorded in T1 and the lowest number of nodules 1.7 plant^{-1} was noted in control T0 (Fig. 2). Again, the number of nodules produced

Table 1. Colony, morphological and biological characteristics of African dhaincha (*Sesbania rostrata*) isolates

Isolate number	SR-R-1	SR-R-2	SR-R-3	SR-R-4	SR-R-5
Pigmentation	Dull white	Dull white	Dull white	Dull white	Dull white
Diameter (mm)	1.1	0.9	1.0	1.2	0.8
Form	Circular	Circular	Circular	Circular	Circular
Margin	Entire	Entire	Entire	Entire	Entire
Surface	Smooth	Smooth	Smooth	Smooth	Smooth
Optical chract.	Translucent	Translucent	Translucent	Translucent	Translucent
Consistency	Viscous	Viscous	Viscous	Viscous	Viscous
Shape	Rod	Rod	Rod	Rod	Rod
Gram	Negative	Negative	Negative	Negative	Negative
Motility	Poorly motile	Poorly motile	Poorly motile	Poorly motile	Poorly motile
Background staining	Transparent	Transparent	Transparent	Transparent	Transparent
Congo red dye absorp.	Slight	Slight	Medium	Medium	slight
Starch utilization	Negative	Negative	Negative	Negative	negative
pH test	Alkali producer	Alkali producer	Alkali producer	Alkali producer	Alkali producer

by T4, T5, T3, and T2 were statistically identical to each other. Vest et al. (1973) reported that Rhizobium isolates differ in their ability to induce nodulation.

The results in Fig. 2 show that all the isolates gave significantly higher number of nodules on the root over control (T0). The highest number of nodules on root plant^{-1} was 59.3 recorded in T5 and the lowest 3.0 plant^{-1} in control. The isolates SR-R-5, SR-R-4 and SR-R-2 were statistically similar to each other but superior to SR-R-3 and SR-R-1 in producing the number of nodules on root. Again, the root nodules produced by SR-R-3 and SR-R-1 were statistically identical to each other.

It was observed that the effect of different isolates on total number of nodules plant^{-1} was almost similar to their effects on stem and root nodulation of *S. rostrata* (Fig. 2). The total number of nodules plant^{-1} ranged from 4.7 in control (T0) to 92.0 observed in T5. The treatment T5, T4 and T2 were statistically identical but superior to all other treatments recording the total number of nodules plant^{-1}. Similarly, the isolates SR-R-2 and SR-R-1 were statistically identical but superior to SR-R-3 where the isolates SR-R-1 and SR-R-3 were statistically similar. Allen and Allen (1981) observed that effective isolates induced large number of nodules around the root system.

3.3 Dry weight of nodule

Stem nodule weight of *S. rostrata* was significantly influenced by inoculation with *Azorhizobium* isolates. The results in Table 2 show that all the isolates had significantly higher dry weight of nodules over control (T0). The highest dry weight of stem nodules was 143.3 mg plant^{-1} recorded in SR-R-4 (T4) and the lowest 33.0 mg plant^{-1} in control (T0). The isolates SR-R-1, SR-R-3, SR-R-5and SR-R-2 were statistically similar to each other but inferior to SR-R-4 in producing the dry weight of stem nodules plant^{-1}. Among them, SR-R-1 and SR-R-3 were also numerically identical. This indicates the high efficacy of the isolate SR-R-4 to fix N_2.

Dry weight of root nodules plant^{-1} was also significant and ranged from 46.7 mg in control (T0) to 126.7 mg observed in SR-R-1 (T1) (Table 2). The dry weight of root nodules of all the isolates was superior to control (T0). The effect of the isolates SR-R-2, SR-R-5 and SR-R-3 on dry weight of root nodules (mg plant^{-1}) was statistically identical but inferior to SR-R-1. Again, there was no statistical difference among the isolates SR-R-2, SR-R-5 and SR-R-3. It was observed that the highest dry weight of root nodules plant^{-1} belonged to SR-R-1 (T1) because of getting comparatively bigger size of root nodules due to inoculation with Azorhizobial isolate.

Results recorded in Table 2 show that total dry weight of nodule of *S. rostrata* was influenced significantly by inoculation with *Azorhizobium* isolates. The total dry nodule weight ranged from 80.0 mg plant^{-1} observed in uninoculated control (T0) to 213.0 mg plant^{-1} recorded in SR-R-1 (T1) which was statistically similar to SR-R-4. The isolates SR-R-1 and SR-R-4 were statistically identical but superior to all other isolates in producing the total dry weight of nodules (mg plant^{-1}). The isolate SR-R-5 was followed by SR-R-2 and SR-R-3 which were statistically identical but superior to control (T0).

3.4 Dry matter yield at 60 DAS

Dry matter yield of shoot was markedly influenced by different isolate inoculation treatments (Table 2). Analysis of variance showed significant effect of different isolates on shoot yields (mg plant^{-1}). Results in Table 2 show that all the isolates gave significantly higher dry matter yield of shoot (mg plant^{-1}) over control (T0). The shoot yields ranged from 1013 mg plant^{-1} in control (T0) to 4553 mg plant^{-1} observed

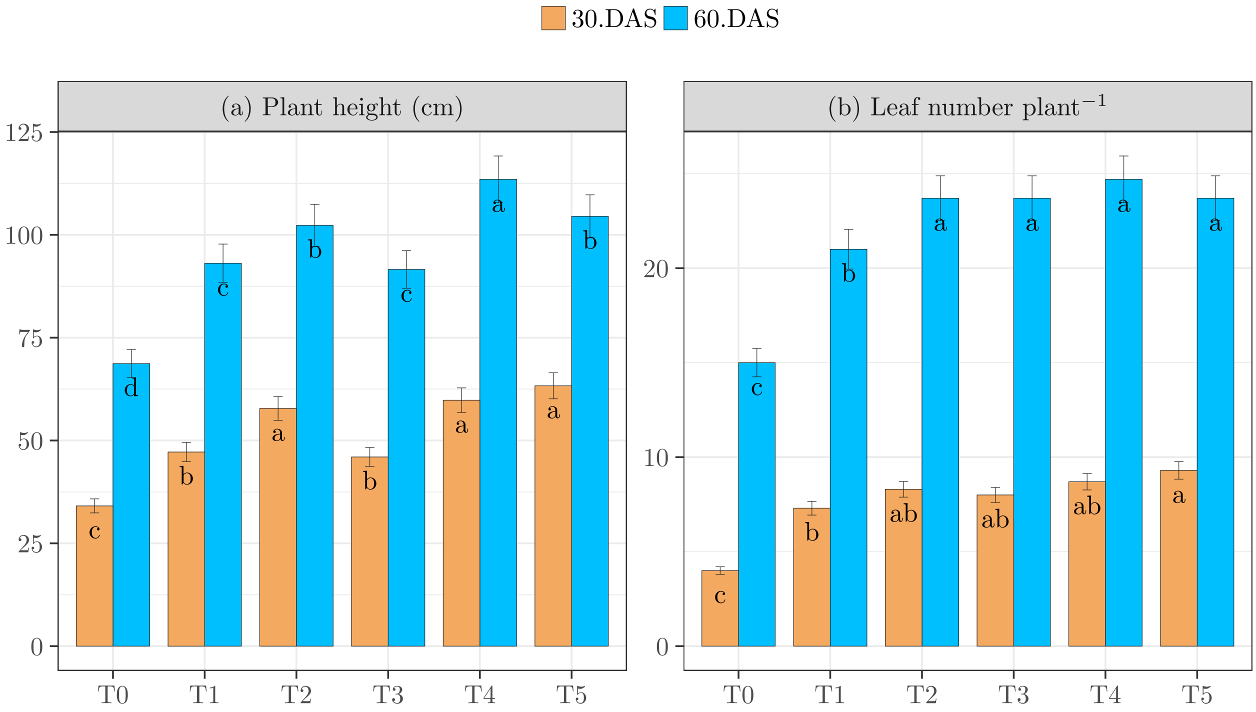

Figure 1. Effects of *Azorhizobium* isolates on plant height and leaf number plant^{-1} of African dhaincha (*Sesbania rostrata*) at 30 and 60 DAS

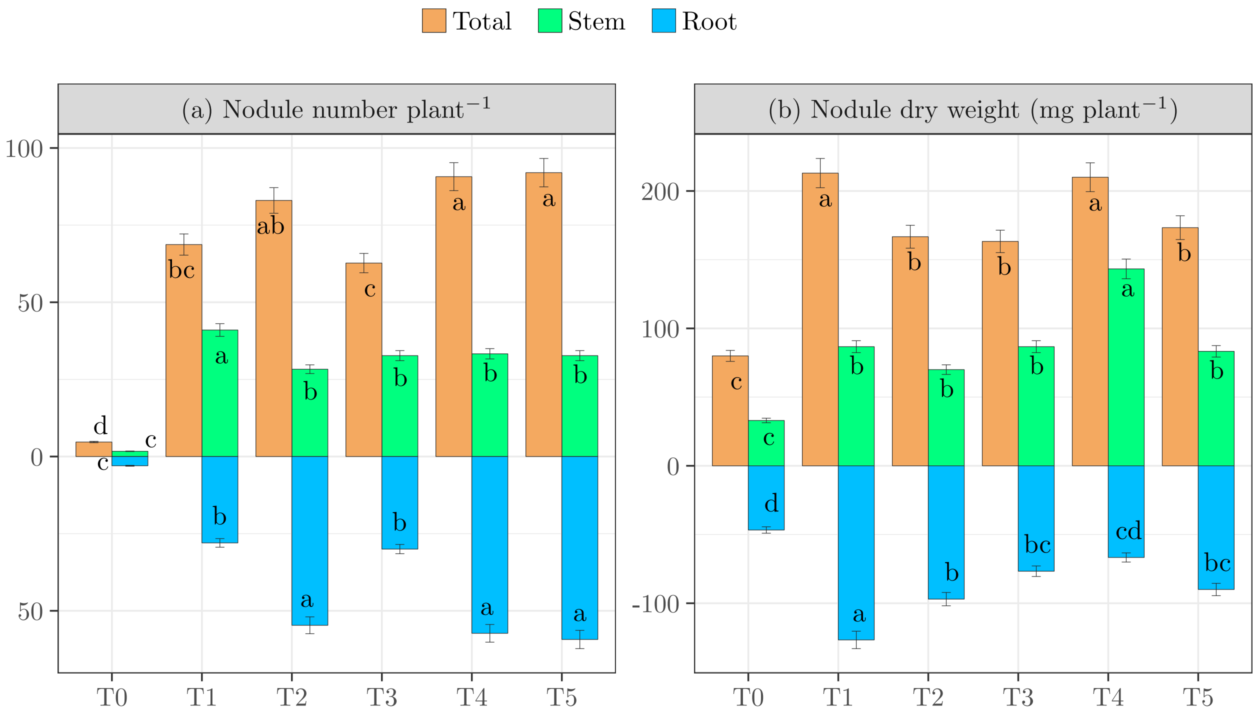

Figure 2. Effects of *Azorhizobium* isolates on nodulation of African dhaincha (*Sesbania rostrata*) at 60 DAS

Table 2. Effects of Azorhizobium isolates on dry matter yield, nitrogen content and nitrogen uptake by shoot of African dhaincha (*Sesbania rostrata*) at 60 DAS

	Dry matter (DM) yield (mg plant^{-1})			%DM ↑	Shoot N	Shoot N	%N uptake ↑
	Shoot	Root	Total	over control	conc. (%)	uptkae (mg plant^{-1})	over control
T0	1013 e	203.3 c	1217 e	–	1.8 d	18.2 d	–
T1	2418 d	480.0 b	2898 d	138	3.5 a	83.7 c	359
T2	3270 c	610.3 b	3880 c	219	3.3 a	108.6 b	496
T3	3587 bc	946.7 a	4533 b	272	2.7 c	96.5 bc	429
T4	4553 a	930.0 a	5483 a	351	2.9 b	133.0 a	629
T5	3813 b	933.3 a	4747 b	290	2.8 bc	107.5 b	490
CV (%)	9.32	14.32	8.85	–	3.0	9.87	–
$S_{\bar{x}}$	167.3	56.55	193.8	–	0.048	5.16	–

in T4. The shoot yields produced by T5 and T3 were statistically similar to each other but higher than that in T1 and in control (T0). Again, shoot yields produced by T3 and T2 was statistically identical but higher than that of isolates T1 and in control (T0). The better shoot yield happened in the isolate T4 was due to high N uptake.

The effect of isolates on the dry matter yield of root was highly significant. Data in Table Table 2 show that all the isolates recorded significantly higher root yield over control (T0). The highest root yields (933.3 mg plant^{-1}) recorded for the isolate SR-R-3 was statistically similar to that of the isolate SR-R-4 and SR-R-5. They all produced significantly higher root yields compared to others. The lowest root yields (203.3 mg plant^{-1}) were recorded in control (T0).

There was a significant effect of different treatments on total dry matter yield of *S. rostrata* (Table 2). The dry matter yield of the crop ranged from 1217 mg plant^{-1} observed in control (T0) to 5483 mg plant^{-1} noted for the treatment T4 (SR-R-4). The total dry matter yield produced by the isolate SR-R-4 (T4) was the highest (5483 mg plant^{-1}) and control produced the lowest dry matter yield (1217 mg plant^{-1}). The isolates SR-R-5 and SR-R-3 produced statistically identical results but they both were superior to the yields produced by the isolates SR-R-2, SR-R-1 and control (T0). It may be noted that the isolate SR-R-4 produced good number of nodules which supported the highest dry matter yield. The percent dry matter increase over control (T0) was 351, 290, 272, 219 and 138 due to inoculation with the isolates SR-R-4 (T4), SR-R-5 (T5), SR-R-3 (T3), SR-R-2 (T2) and SR-R-1 (T1), respectively.

3.5 Nitrogen content of shoot at 60 DAS

Results reported in Table 2 indicate that N content in the shoot of *S. rostrata* was influenced significantly by inoculation. The N content of shoot ranged from 1.80% recorded in control (T0) to 3.46% recorded in SR-R-1 (T1). The treatments T1 and T2 gave significantly higher N content of shoot over all other treatments. Again, inoculation with SR-R-4 and SR-R-5 recorded statistically identical N content in shoot. The isolates SR-R-4 and SR-R-5 were followed by SR-R-3 and control (T0).

3.6 Total N uptake by shoot

Analysis of variance showed significant effect of different treatments on N uptake by shoot of the crop *S. rostrata* (Table 2). In the present study, the highest N uptake by shoot was 133.0 mg plant^{-1} which was obtained with the isolate SR-R-4 and the lowest was 18.2 mg plant^{-1} in control (T0). The treatment T1 recorded significantly higher N uptake by shoot compared to all other treatments. The isolates SR-R-2, SR-R-5 and SR-R-3 were found statistically similar to each other in case of N uptake by shoot. The increased N uptake due to inoculation of isolates was presumably due to high rate of N_2-fixation. This indicated the efficacy to the isolate. Sattar and Podder (1988) showed that seed inoculation with *Rhizobium* increased nitrogen fixation by chick pea germ plasm and recorded maximum nitrogen fixation in G-97 (73 kg N ha^{-1}) followed by G-296 (70 kg N ha^{-1}) and ICCT-5 (65 kg N ha^{-1}) and the percent increase over control being 188, 126 and 69, respectively.

4 Discussion

The principal objective of this research was also to select effective Azorhizobial isolates for using them as the inoculants for producing better green manuring crops. *Azorhizobium* was isolated from stem nodules of *S. rostrata*. Five isolates were identified as *Azorhizobium* on the basis of colony, morphological and biochemical characteristics. But finally nodulation test was carried out to confirm them as Azorhizobial isolates and their efficacy. Inoculation of the isolates produced huge number of nodules compared to no inoculation *i.e.* control. For example, the stem, root and total nodule number of the inoculated plants of the present study was higher than uninoculated control (T1).

The highest total nodule number (92.0 plant^{-1}) was recorded for inoculation of SR-R-5 (T6) whereas the lowest (4.7 plant^{-1}) for no inoculation *i.e.* control. The increased nodule number due to *Azorhizobium* inoculation has been reported by many other workers (Yang et al., 1998; Ndoye et al., 1994; Balasubramani and Kannaiyan, 1993; Ladha et al., 1989). Dry matter yields due to inoculation were also higher compared to that of uninoculated control plants. For example, the highest total dry matter yield (5483 mg plant^{-1}) was recorded for inoculation of SR-R-4 whereas the lowest (1217 mg plant^{-1}) value was obtained for no inoculation treatment *i.e.* control. Inoculation of the isolates had significant effect on N content of shoot and total N uptake by shoot. The highest N content of shoot (3.5%) was recorded for inoculation with SR-R-1 but the highest total N uptake by shoot (133.0 mg plant^{-1}) was recorded for inoculation of SR-R-4 whereas the lowest N content of shoot (1.8%) and N uptake by shoot (18.2 mg plant^{-1}) were observed for the un-inoculated control plants.

The highest plant height at 60 DAS was also observed in inoculated plant (T4) whereas the lowest in the un-inoculated control (T0). The increased N content shoot, N uptake by shoot, dry matter yield, plant height was due to inoculation presumably came from N_2-fixation that produces from the microbial relationship between plants and microbes (Dahmardeh, 2013; Ella, 2006). Several research workers have also reported that *Azorhizobium* inoculation increased N content of shoot and total N uptake by shoot (Dreyfus et al., 1985; Hussain and Ibrahim, 1987; Ventura et al., 1987).

5 Conclusions

Considering all the growth parameters, nodulation, dry matter yield, N content of shoot and total N uptake by shoot, it may be inferred that the isolate SR-R-4 was the best. The isolates SR-R-5 and SR-R-3 were also found promising. Further studies are necessary to establish the promising strains of *Azorhizobium* for cultivation of *Sesbania rostrata*.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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